

expression vectors to produce portions of pp28 that immunologically bind to antibodies against pp28. (See specification at p. 2-3.) These same methods can and have been used to obtain prokaryotic expression vectors encoding HCMV pp28 in strains other than Ad169 of HCMV. *Pande* 1991, of record and *Pande* 1988, attached, although not prior art, are evidence that the same techniques disclosed in the specification enabled *Pande* to obtain a prokaryotic expression vector encoding HCMV pp28 from HCMV Towne strain. (See 1991 *Pande* at 762, and 1988 *Pande* at 306-307.) Reference to the post-filing date *Pande* articles is not improper, since they are not being offered as evidence to supplement the disclosure, but rather are being offered as evidence of the level of ordinary skill in the art at the time of the application and as evidence that the disclosed invention would have been operative. *Gould v. Quigg*, 3 U.S.P.Q.2d 1302, 1305 (Fed. Cir. 1987). Therefore, Applicants request that this rejection be withdrawn.

II. 35 U.S.C. §112, Second Paragraph

The Office has rejected claims 19, 21, 24-36, 38, and 40 under 35 U.S.C. §112, second paragraph, as allegedly being indefinite. (Office Action at page 3-4.) In particular, claim 19 was rejected for reciting "said DNA molecule does not comprise an entire HindIII R fragment from the genome of human cytomegalovirus strain Ad169." The Office believes that the metes and bounds of the claim are not entirely clear, "because it is not clear what the intended DNA does comprise and what the claim encompasses." The Office also finds the claim language confusing as to what is being

claimed in terms of either strain Ad169 or "all HCMV strains." Similarly, the Examiner believes that the metes and bounds of claims 25-36 remain unclear for the same reasons. Applicants have amended the claims 19, and 21, and cancelled claims 24-36, 38, and 40 to more particularly point out and distinctly claim the subject matter Applicants regard as their invention. Therefore, Applicants request that this rejection be withdrawn.

III. 35 U.S.C. §102(b)

The Office has newly rejected claims 19, 21, and 25 under 35 U.S.C. §102(b) as anticipated by *Ihara et al.*, interpreted in light of *Pande et al.* (Office Action at page 4-5.) The Office argues that *Ihara* anticipates, because its restriction fragment DNA molecules inherently contain sequences encoding pp28 or parts thereof and do not contain the HindIII fragment of Ad169 since they do not originate from Ad169. Applicants have amended claims 19, and 21, and cancelled claim 25 to recite a prokaryotic expression vector or a prokaryotic or eukaryotic cell transformed with a recombinant DNA molecule that expresses HCMV pp28. Therefore, Applicants request that this rejection be withdrawn, because *Ihara* does not disclose the expression of HCMV pp28 from an expression vector.

Applicants respectfully request allowance of the amended and newly proposed claims. Alternatively, if the Office believes that the claims as proposed are not allowable Applicants would like to schedule an interview with the Examiner.

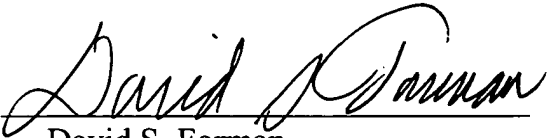
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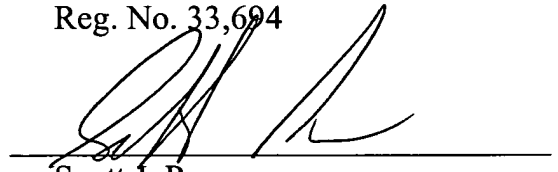
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Respectfully submitted,

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**APPENDIX TO REQUEST FOR SECOND EXAMINATION AFTER
FINAL UNDER 37 C.F.R. § 1.129(a) AND AMENDMENT**

IN THE CLAIMS:

19. (Four Times Amended) A [DNA molecule] prokaryotic expression vector encoding HCMV pp28[,] or antigenic [portions] portion thereof that [elicit] elicits antibodies that immunologically bind to pp28, wherein said [DNA molecule does not comprise an entire HindIII R fragment from the genome of human cytomegalovirus strain Ad169] vector expresses said HCMV pp28 or antigenic portion thereof in prokaryotic cells.

21. (Four Times Amended) A prokaryotic cell which is transformed with a recombinant DNA molecule encoding HCMV pp28, or antigenic [portions] portion thereof that [elicit] elicits antibodies that immunologically bind to pp28, wherein said cell expresses said HCMV pp28 or said antigenic portion.

37. (Two Times Amended) [The] An isolated 0.5 kB KpnI/SmaI fragment encoding an antigenic [portions] portion of pp28 that [elicit] elicits antibodies that immunologically bind to pp28 [, within the HindIII R fragment from the genome of human cytomegalovirus].

39. (Two Times Amended) [The] An isolated 1.0 kB SmaI/SmaI fragment encoding an antigenic [portions] portion of HCMV pp28 that elicit antibodies that

immunologically bind to pp28 [, within, but not including, the HindIII R fragment from the genome of human cytomegalovirus].

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Genomic Localization of the Gene Encoding a 32-kDa Capsid Protein of Human Cytomegalovirus

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We have determined the map position of a viral gene encoding a 32-kDa late structural protein of human cytomegalovirus (HCMV) using a murine monoclonal antibody. This monoclonal antibody was reactive with two protein bands of 32 and 27 kDa in HCMV-infected cell lysates and with a single 32-kDa protein band in HCMV virions as detected by immunoblot analysis. When purified HCMV envelope preparation was used for immunoblotting, the monoclonal antibody did not display a detectable band. We used this monoclonal antibody to screen a cDNA library that was constructed from poly(A)⁺ RNA of late HCMV-infected cells and cloned into the expression vector λ gt11. A cDNA clone that expressed an immunoreactive epitope of the late HCMV protein fused to β -galactosidase was identified. Probing the restriction digests of HCMV (Towne and AD169) DNA with insert DNA from the immunoreactive λ gt11 clone permitted us to localize the coding sequence within the long unique region between map coordinates of 0.62 and 0.64 of HCMV Towne and AD169 genomes. Using the same probe, a single transcript of 1.4 kb was detected in total RNA from HCMV-infected cells at late times after infection. © 1988 Academic Press, Inc.

Human cytomegalovirus (HCMV) is associated with a variety of infectious syndromes in humans (1). Although most HCMV infections do not result in clinical illness, a significant number of immunocompromised individuals, such as bone marrow transplant recipients and individuals with acquired immunodeficiency syndrome (AIDS), develop severe and sometimes life-threatening disease associated with HCMV (2, 3). HCMV is the largest herpesvirus, with a linear double-stranded genome of about 235 kilobase pairs (kb) that codes for at least 35 structural proteins with molecular sizes ranging from 15 to >200 kDa (4-7). Several of these viral proteins are modified post-translationally by glycosylation or phosphorylation and appear to be important for determining the host's immune response to HCMV infection (8-15). A number of these virion-associated proteins have been identified and mapped by screening expression libraries with monoclonal and polyclonal antibodies (16-19).

To isolate and characterize viral genes encoding antigenic components of HCMV we have constructed cDNA and genomic libraries of HCMV (Towne) DNA in phage expression vector λ gt11. In this communication we describe a monoclonal antibody (Mab) to a 32-kDa capsid protein of HCMV and its use for the isolation and subsequent mapping of viral coding sequences on HCMV Towne and AD169 genomes.

Hybridomas were prepared from mouse splenic lymphocytes after immunization with purified Towne strain

of HCMV virions plus dense bodies. The hybridomas were screened for anti-HCMV antibody production by enzyme-linked immunoassay (ELISA) using the original virion immunization reagent absorbed to microtiter plate wells. Positive hybridoma lines were cloned three to five times by limiting dilution and stable antibody producing subclones were grown in ascites form. One Mab (Mab-48) was selected for further characterization based upon results of immunoblot assays. Lysates of HCMV-infected or uninfected human foreskin fibroblasts (HFF) were prepared by sonication and used for immunoblotting. SDS-polyacrylamide gel electrophoresis was conducted using 10% polyacrylamide slab gels and 10 μ g of antigen per well. The proteins were transferred electrophoretically from gels to nitrocellulose membranes and the immunoreactive bands were detected by treating nitrocellulose membranes with murine Mab followed by second antibody staining with goat anti-mouse horseradish peroxidase conjugate and chloronaphthol substrate. Mab-48 reacted strongly with two protein bands of 32 and 27 kDa from HCMV-infected cells but not uninfected controls (Fig. 1A).

To further investigate the morphological distribution of the polypeptides detected by Mab-48, HCMV virions and the HCMV envelope proteins were purified using previously described standard procedures (7, 20). Briefly, the HCMV virions were isolated from extracellular viral particles using a 20-70% sucrose gradient at 25K rpm for 1 hr. The virus envelope proteins were solubilized by suspending the purified virions in 50 mM Tris-buffered saline, pH 7.2 (TBS), containing 1% Non-

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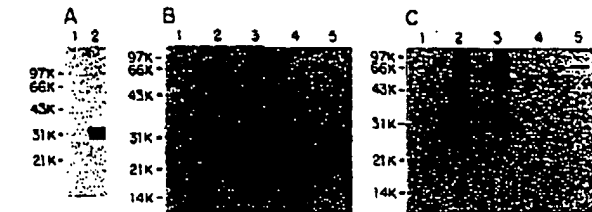


Fig. 1. Immunoblot analysis of HCMV proteins with Mab-48 and a rabbit antiserum to HCMV virions. HCMV proteins were analyzed by electrophoresis on either a 10% SDS-polyacrylamide gel (A) or 12% SDS-polyacrylamide gels (B and C). After electrophoretic transfer to nitrocellulose filters, (A) and (B) were treated with Mab-48 and filter (C) was treated with a rabbit antiserum to HCMV. Lanes 1, protein lysate from uninfected HFF cells; 2, protein lysate from HCMV-infected HFF cells; 3, HCMV virion proteins; 4, HCMV envelope proteins; and 5, HCMV core proteins. Immunoreactive bands were stained with peroxidase-conjugated second antibody and chloronaphthol substrate.

idet-P40 (NP-40) for 1 hr at 4°. The detergent-insoluble virion core fraction was removed by pelleting through a 15% sucrose cushion at 25K rpm for 1 hr. The purified intact virions, the supernatant fraction containing NP-40-soluble envelope proteins, and the pelleted virion core particles were then used as antigens in an immunoblot assay, as described above, except that a 12% SDS-polyacrylamide gel was used to separate the proteins. Intact virions and the virion core particles displayed only one prominent band of 32 kDa that was stained by our immunoassay with Mab-48 (Fig. 1B). The 27-kDa protein detected in HCMV-infected cells is probably a precursor or a proteolytic fragment of the 32-kDa protein. The 32-kDa protein band was also detected when a rabbit antiserum to HCMV virions was used for immunostaining (Fig. 1C). The virion envelope fraction exhibited a relatively weaker reaction by immunostaining (Fig. 1B) indicating that the 32-kDa protein is primarily associated with the virion core. This Mab was used for screening a cDNA library prepared from mRNA of late HCMV-infected HFF cells.

Total cytoplasmic RNA was extracted from HCMV Towne-infected HFF cells 90 hr postinfection using the procedure of Daskal *et al.* (21). Poly(A)-containing RNA was isolated by chromatography over an oligo(dT)-cellulose column (PL Biochemicals) and cDNA was synthesized from 5 µg of poly(A)⁺ RNA essentially as described by Mostov *et al.* (22). Double-stranded cDNA was rendered blunt ended with T4 polymerase and then modified with T4 kinase to ensure that all the 5' ends of the cDNA molecules were phosphorylated. For convenient cloning into λgt11, asymmetrical *Eco*RI linkers, also containing a *Cla*I site, were added to ends of cDNA by blunt end ligation (23). The sequence of *Eco*RI linkers was

5'-AATTCGGTATCGATGTGC
GGCATAGCTACACG-5'.

The *Cla*I site is highlighted. Initially, only the 5' end of the shorter strand (14 nucleotides) was phosphorylated, and a 100-fold molar excess of adaptors (phosphorylated 14-mer and 5'-hydroxyl 18-mer) over calculated cDNA ends was used for the ligation reaction. Subsequently, the linked cDNA was phosphorylated with T4 kinase and the excess unligated linker oligomers were removed by chromatography over a Sepharose CL4B column (Sigma). The cDNA was cloned into λgt11 arms prepared by digesting λgt11 DNA with *Eco*RI followed by treatment with calf intestine alkaline phosphatase. After *in vitro* packaging of the phage heads, a library of approximately 0.9×10^6 independent recombinant clones was obtained. The library was screened with Mab-48, described above, using the modified method of Young and Davis (24) in which horseradish peroxidase-conjugated second antibody (Cappel) and 4-chloro-1-naphthol (Aldrich) were used for detection of bacteriophage clones expressing immunoreactive epitopes of 32-kDa HCMV protein fused to β-galactosidase. Approximately 1.5×10^5 clones were screened with Mab-48 and three positive signals were obtained. Characterization of one clone, λ21121 which also gave a positive reaction with immune antiserum obtained from a bone marrow transplant recipient following HCMV infection (data not shown), is described here.

Restriction analysis of phage DNA from plaque purified clone λ21121 with *Eco*RI revealed the size of the cDNA insert to be approximately 350 bp. The insert DNA was isolated by preparative agarose gel electrophoresis using low-melting-point agarose and then used for Southern blot analysis of HCMV DNA. HCMV Towne and AD169 DNAs were digested with restriction enzymes *Eco*RI, *Bam*HI, *Hind*III, *Xba*I, and *Pst*I, electrophoresed through a 0.6% agarose gel, and transferred to nitrocellulose membranes. When these blots were probed with ³²P-labeled 350-bp insert DNA from clone λ21121, strong hybridization was detected to a single HCMV fragment in each restriction digest (Fig. 2A). The probe hybridized with the following series of HCMV restriction fragments: *Eco*RI-B, *Bam*HI-D, *Hind*III-B, and *Xba*I-C of HCMV Towne; and *Eco*RI-B, *Bam*HI-B, *Hind*III-R, and *Xba*I-F of HCMV AD169 (Fig. 2A). In addition, strong hybridization was detected to a single *Pst*I fragment having a size of 5.3 kb in both HCMV Towne and AD169. The 5.3-kb hybridizing fragment corresponds to the *Pst*I-J fragment in HCMV AD169 (25). These data indicate that the gene encoding the 32-kDa structural protein is present in both HCMV Towne and AD169 strains and maps to an iden-

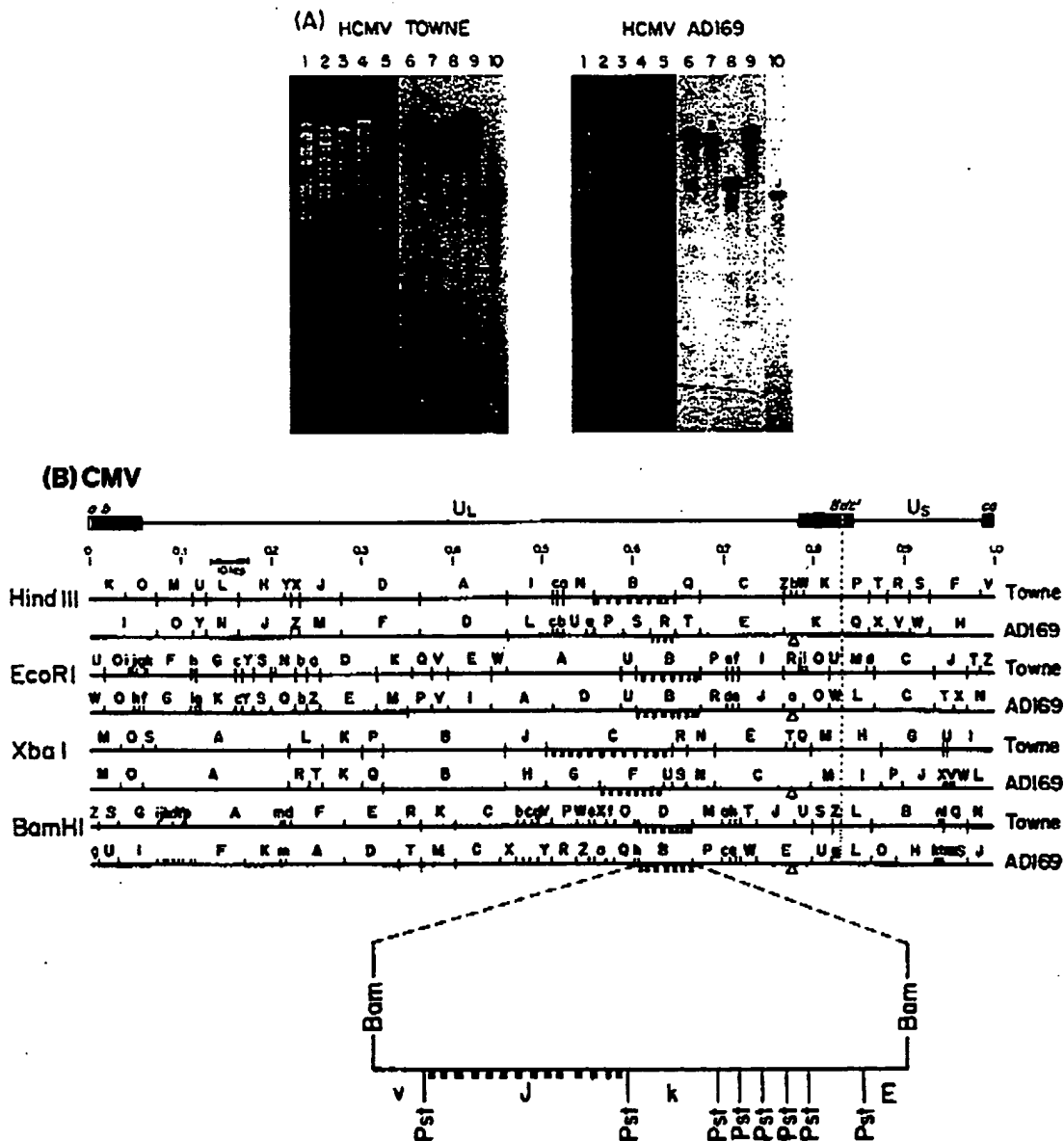


FIG. 2. Hybridization analysis and genomic mapping of the 350-bp HCMV cDNA fragment from the immunoreactive clone λ 21121. (A) Southern blot hybridization of the 350-bp cDNA fragment to HCMV Towne and AD169 DNA restriction fragments. The restriction fragment profiles of HCMV Towne and AD169 DNAs digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Hind*III (lane 3), *Xba*I (lane 4), and *Pst*I (lane 5) as visualized by ethidium bromide staining and the corresponding autoradiographic images after hybridization (lanes 6–10) are shown. Hybridization was conducted in a mixture containing 6X SSC, 10 mM EDTA, 5X Denhardt's solution, 0.5% SDS (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate) at 65° for 18–18 hr. Filters were then washed with 1X SSC/0.5% SDS three times, 15 min each at room temperature followed by two 60-min washes at 68°. The letters on each lane designate the hybridizing fragment in each digest. (B) Restriction map of HCMV (Towne) and HCMV (AD169) genomes and localization of the cloned HCMV gene. The compiled restriction maps for *Hind*III, *Eco*RI, *Xba*I, and *Bam*HI for HCMV (Towne) and (AD169) were taken from a recent report of Kemble *et al.* (19). The top line indicates a schematic representation of the HCMV genome with unique sequences (U_L and U_S) and inverted repeat regions of the L and S components. Genomic map units are shown between the top line and the restriction maps. Restriction fragments that hybridized to the 350-bp DNA fragment shown in (A) are denoted on each restriction map by thick vertical lines facing downward. The expanded region below the genomic restriction maps indicates the position of the 5.3-kb *Pst*I restriction fragment carrying homologous sequences. The *Pst*I restriction map of this region is taken from a publication of Greenaway *et al.* (25).

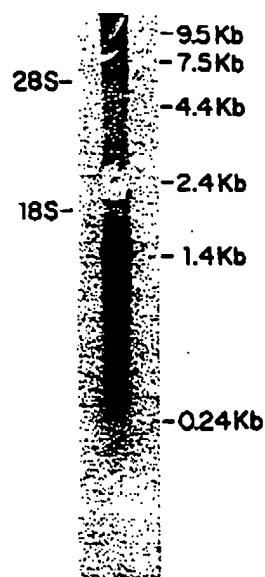


Fig. 3. Northern blot hybridization of ^{32}P -labeled cDNA insert from clone $\lambda 21121$ with whole cell RNA extracted from HFF cells late after HCMV infection. RNA was purified 10 days postinfection and fractionated by electrophoresis on a 1.3% agarose gel containing formaldehyde. The gel was blotted onto GeneTrans-45 membrane and probed with ^{32}P -labeled 350-bp cDNA insert from clone $\lambda 21121$ in a mixture containing 50% deionized formamide, 3X SSC, 5X Denhardt's solution, 1.0% SDS, and 5% dextran sulfate at 42° for 16–18 hr. Washing was in 0.1X SSC/0.1% SDS, three times, 20 min each at room temperature followed by two 20-min washes at 52°. Molecular weight standards include 0.24- to 9.5-kb RNA ladder (BRL) and human cell rRNAs of 28 S (5.2 kb) and 18 S (2.0 kb).

tical position at approximately 0.62–0.64 map units in the long unique region of both virus strains (Fig. 2B).

Northern blot analysis was performed to determine the size of the transcript for this virion protein. Total late RNA from infected HFF was electrophoretically separated and transferred to GeneTrans-45 (Plasco). When this blot was probed with ^{32}P -labeled insert DNA from clone $\lambda 21121$, a single transcript was detected (Fig. 3). The size of this transcript was estimated to be approximately 1.4 kb, based on RNA size standards (BRL).

At least three HCMV proteins having molecular weights in the range of 25–35 kDa have been described by several investigators. Immiere and Gibson (26, 27) have shown the presence of a 34-kDa minor capsid protein in extracellular and intranuclear viral particles including the mature virion, the noninfectious enveloped particle (NIEP), and the A- and B-capsids found within the nucleus of HCMV-infected cells. Interestingly, they found a 28-kDa protein associated with both types of intranuclear capsids that is not present in NIEPs or mature virions. These findings are consistent with our data showing the presence of 32- and 27-kDa

proteins in extracts from infected cells, while the 32-kDa moiety was predominant in extracellular virions and virion core particles.

There are several reports of murine Mabs that appear to recognize HCMV proteins of approximately the same size as those detected by Mab-48. Re *et al.* (28) have described a Mab that recognizes a structural protein of 28 kDa, which is present in both the cytoplasm of infected cells during the late phase of the viral replication cycle and in extracellular viral particles. Using immunoelectron microscopy, the 28-kDa structural protein was localized to the surface of cytoplasmic capsids, but not dense bodies or intranuclear capsids (29). Similarly, Nowak *et al.* (10) have characterized a virion-associated phosphoprotein of 29 kDa that appears to be a component of the HCMV capsid. Pereira *et al.* (30) have described several Mabs that recognize a family of four glycoproteins designated gD1 through gD4. Two lower molecular weight species of 34 and 25 kDa are similar in size to the proteins detected by our Mab-48. However, as our Mab fails to detect the higher molecular weight species of the gD family, the 32- and 27-kDa polypeptides detected by Mab-48 are probably distinct from gD3 and gD4. In addition, while Mabs to the gD family stain the surface of intact HCMV-infected cells, suggesting these glycoproteins are membrane-associated, substantial amounts of the 32- and 27-kDa proteins were not detected in the viral envelope using Mab-48.

In summary, we have localized the gene encoding a 32-kDa structural protein of HCMV, which most likely corresponds to the minor capsid protein described by others (10, 26, 28). It has been demonstrated that a protein of this size is recognized by most human sera possessing anti-HCMV antibodies (8–15). Since the clone $\lambda 21121$ gave a positive reaction with HCMV convalescent human sera, it is likely that it encodes one of the immunodominant proteins to which antibodies are produced during natural HCMV infection. Further studies, including expression cloning, can now be initiated for a more detailed investigation into the structural, functional, and antigenic properties of the protein.

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